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Award Number: W81XWH-07-1-0136

TITLE: Tulane/Xavier Vaccine Development/Engineering Project

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REPORT DATE: August 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2011	Annual	1 February 2010 – 30 June 2011

4. TITLE AND SUBTITLE

Tulane/Xavier Vaccine Development/Engineering Project

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0136

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Tulane University School of Medicine
New Orleans, LA 70112

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The Tulane/Xavier Biodefense Vaccine Development/Engineering project will develop new vaccines against biological threat agents to aid the war-fighter. Through the innovative use of nanotechnology, researchers and engineers from the Tulane University Schools of Medicine and Science & Engineering and the Xavier College of Pharmacy will fabricate nanoparticulate systems that are effective for transdermal and mucosal delivery of life-saving vaccines. One aim of this project will be to compare different nanocarriers (i.e., nanohydrogels, star copolymers, and spray-dried PLGA nanoparticles) for the ability to incorporate biological threat-relevant vaccine antigens and deliver those antigens through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum. The use of nanocarriers for vaccine delivery is a platform technology, applicable to delivery of a variety of existing and potential vaccines.

15. SUBJECT TERMS
Vaccines, Bioterrorism, Biothreat, Nanocarrier, Immunity, Prophylaxis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	8	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

Infectious diseases remain one of the leading causes of death in adults and children world-wide. Each year, infectious diseases kill more than 17 million people, including 9 million children. In addition to suffering and death, infectious diseases impose an enormous financial burden on society. Although antibiotics and vaccines have been effective at reducing the morbidity and mortality of some infectious diseases, new ones such as AIDS, Lyme disease, West Nile fever, Hanta virus, SARS, and Avian Influenza virus are constantly emerging, while others such as malaria and tuberculosis reemerge in drug-resistant forms. Furthermore, we have an aging adult population with diminishing immune function, increased use of immunosuppressive agents for cancer, tissue transplantation, and autoimmune disease, and an upwardly spiraling cost of health care delivery that makes some existing vaccines unaffordable by the populations at greatest risk. In addition, we now face the possibility of bioterrorism with potentially devastating consequences and a limited number of preventative and therapeutic options.

A great deal of effort has been directed towards developing nonparenteral (needle-free) alternatives to traditional vaccine delivery. Nonparenteral vaccines offer a number of potential advantages over traditional vaccines including 1) the potential to confer mucosal as well as systemic immunity, 2) increased stability, 3) increased shelf-life, 4) elimination of needles and the need for specially trained healthcare specialists to administer vaccines, and 5) potentially lower costs. One such approach, transcutaneous immunization (TCI), is a non-invasive, safe method of delivering antigens directly onto bare skin. Immunization is achieved by direct topical application of a vaccine antigen. Despite the attractiveness of TCI, the technology is limited by the relative inefficiency of transport of large molecular weight vaccine antigens across intact skin.

Recent innovations in transdermal delivery of drugs, including chemical enhancers, electricity, ultrasound, and microneedles, demonstrate the feasibility of large-molecule transport through the skin's permeation-barrier, specifically the stratum corneum. This outer layer of the skin is composed of tightly packed lipid molecules and the dense, crystalline arrangement of these lipids creates the essential barrier to prevent water loss and pathogen entry. Recent evidence has shown that this barrier can be overcome by properly structured nano-sized particles (nanocarriers). This proposal will compare different nanocarriers for the ability to incorporate a model vaccine antigen and deliver that antigen through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum. While the immediate objective will be to deliver vaccines against biological threat agents, the technologies created will have a tremendous impact on health and human welfare around the world because of their applicability to a wide range of infectious diseases and therapeutic treatments, including other infectious diseases that pose threats to the war-fighter and civilian populations.

BODY

Through the innovative use of nanotechnology, researchers and engineers from the Tulane University Schools of Medicine and Science & Engineering and the Xavier College of Pharmacy will fabricate nanoparticulate systems that are effective for transdermal and mucosal delivery of life-saving vaccines. We will compares three different nanocarriers (nanohydrogels, star copolymers, and spray-dried PLGA nanoparticles) for the ability to incorporate a model vaccine antigen and deliver that antigen through the stratum corneum to immune-responsive cells in the epidermis. The specialized assembly of each type of nanocarrier gives each unique properties and different interactions within the lipid channels of the stratum corneum.

In the last funding cycle, we demonstrated that when a stable double emulsion is prepared at a temperature where all three phases – W_1, O, W_2 – are liquid, and then is brought to a lower (storage) temperature where the oil phase – O – freezes, stability is preserved. We also extended our findings on double emulsions and began investigations on nanoscale unimolecular reverse micelle (URM) carriers, and gel systems that are crystalline mesophases. Finally, we began immunization studies with BSA as a model antigen admixed with ceramide liposomes, water-in-oil-in-water (W1/O/W2) emulsions, tubular liposomes, and silica-tube nanogels.

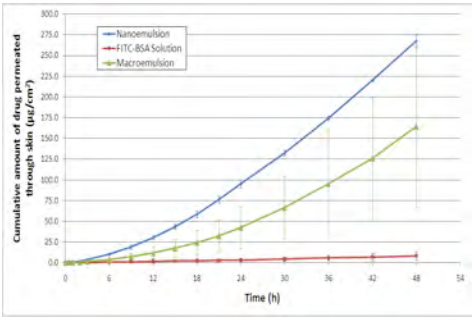
During the first part of this year, we prepared twenty different formulations of poly(lactide-co-glycolide) (PLGA) micro/nanocapsules containing BSA. However, none of these formulations were useful for the delivery of vaccine through skin because of their relatively larger size. We then developed five formulations of nanoemulsions containing FITC-BSA with an average particle size of less than 100 nm, which was ideal for permeation through skin.

The nanoemulsion formulation was optimized according to its particle size, viscosity, and temporal stability. The final formulation chosen contained 55% v/v aqueous phase, 40% v/v squalane as the oil phase, and 5% v/v surfactant/co-surfactant blend composed of Tween-80:Span-80 in 1:1 v/v ratio. The permeation profile of each formula is shown below and is summarized in Table 1. As expected, the FITC-BSA solution showed little penetration due to the barrier function of the stratum corneum. As evident in the graph, the nanoemulsion formulation produced the highest drug permeation after 48 hours. Table 1 shows that the nanoemulsion had the highest steady state flux and a higher enhancement ratio than the macroemulsion solution. Additionally, the nanoemulsion produced consistent results compared to the wide distribution observed for the macroemulsion plot. This result is also reflected in the standard deviation of the steady state flux, which is higher for the macroemulsion. The inconsistency of the macroemulsion is due to the wide range of emulsion particle sizes present in the sample, compared to the small particle size and polydispersity index of the nanoemulsion. Thus, the improved permeation of the nanoemulsion FITC-BSA through the skin compared to FITC-BSA solution can be partly attributed to the size of the nanoemulsion.

Table 1. Permeation Parameters of Different Emulsion Formulations Through Mouse Skin

Formulation	Particle Diameter (nm, ± SD)	Polydispersity Index (± SD)	Jss (μg/cm2hr, ± SD)	Er (relative to FITC-BSA solution)	r2 of linear portion of permeation plots
Nanoemulsion	144.9 (5.1)	0.207 (0.003)	3.994 (0.223)	36.64	0.9975
Macroemulsion	62.0 (27.8) 105.2 (263.0)1	0.256 (0.118)	2.735 (1.767)	25.09	0.9909

1 bimodal size distribution



Permeation profile of cumulative amounts of FITC-BSA permeated through mouse skin for different formulations.

Our custom diffusion cell design has allowed us to accurately measure the skin permeability of our optimized nanoemulsion formulation. This study shows that our nanoemulsion formulation has significant potential as a transcutaneous vaccine delivery system with 36 times greater skin diffusion compared to FITC-BSA solution. Additionally, the nanoemulsion formulation shows consistent, superior results compared to the larger particle size emulsion, demonstrating the impact of manufacturing technique and particle size on the efficacy of transcutaneous formulations. Future work will include determining the exact enhancement effect of the individual components of the nanoemulsion formulation to further optimize the skin permeability of the formulation.

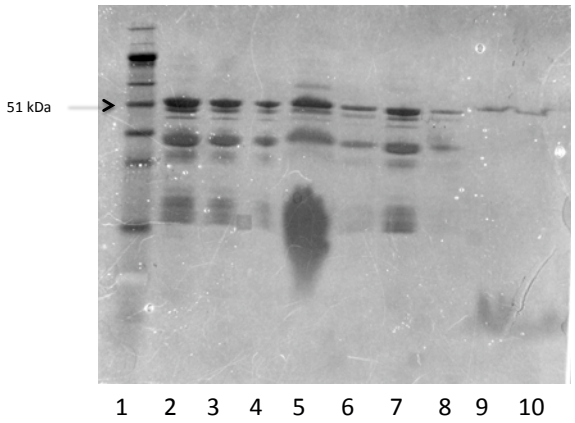
F1-V Purification

One technical problem we encountered was availability of sufficient quantities of endotoxin free F1-V for the proposed studies. We had obtained the plasmid for production of F1-V from our collaborators at USAMRIID and developed a process for purifying the fusion protein from inclusion bodies in recombinant *E. coli*. However, the yield was insufficient to create a homogenous pool of purified material from which we could remove endotoxin (a confounding variable in immunization studies). Our best production was 5 – 10 mg per batch and we estimated that we would need a minimum of 150 mg for the incorporation and immunization studies. We inquired about purchasing the material from our collaborators at USAMRIID, but the price we were quoted ($1,500 per 0.5 mg) was not supportable. Consequently, we undertook a process improvement project (changing promoters and production strains, improving purification techniques) and can now make sufficient quantities of purified material for our studies.

F1-V incorporation

We are in the process of incorporating F1-V into each of the nanocarriers in preparation for the immunization studies. In each case, incorporation will first be evaluated *in vitro* prior to immunization. We initiated these studies with ceramide 3 liposomes encapsulating F1-V. First, a high pH buffer suitable for F1-V was used with FITC-labeled BSA to confirm encapsulation of the protein within the liposome vesicles and to determine the effectiveness of ultracentrifugation to concentrate the dispersion. A significant difference in the fluorescence intensity between the supernatant and the pellet containing the liposome vesicles indicated that the vesicles did indeed contain FITC-BSA. Liposome preparations encapsulating F1-V were prepared and the entrapment and antigenicity of the molecule was indicated by SDS-Page and Western blot analysis, as discussed below. Further ultracentrifugation cycles will allow us to more efficiently remove the unencapsulated antigen until the supernatant is free of detectable protein.

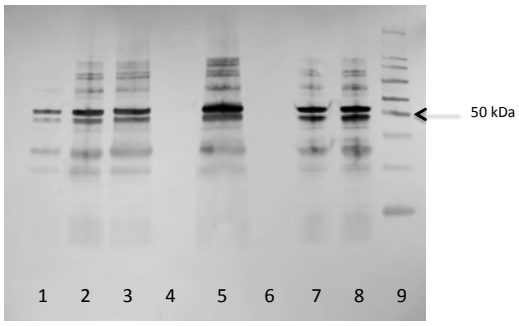
The following formulations containing F1-V (molecular weight = 53 kDa) were prepared: ceramide 3 liposomes, squalene nanoemulsions, and water-in-oil-in-water double emulsions. These samples were analyzed with SDS-Page gel and Western blot to determine whether any degradation of the protein occurred during the preparation of the formulations and whether the protein retained its antigenicity (the ability to be recognized by antiserum to F1-V). A representative SDS-page gel with these formulations is shown below:



The following components were loaded into each lane:
1) molecular weight marking standard
2) 7.5 µg F1-V in aqueous buffer
3) 3.75 µg F1-V in aqueous buffer
4) 1.5 µg F1-V in aqueous buffer
5) 6.67 µ g F1-V encapsulated in water-in-oil-in-water double emulsion formulation
6) 15 µ L of liposome pellet collected after ultracentrifugation
7) 3.75 µg F1-V in ceramide 3 liposome (initial preparation before ultracentrifugation) in a total volume of 5 µL
8) 5 µL supernatant after centrifugation of ceramide 3 liposomes
9) F1-V encapsulated in nanoemulsion formulation with protein added prior to emulsification and homogenization
10) F1-V encapsulated in nanoemulsion formulation C with protein added after emulsification and homogenization

From these studies we concluded that 1) There is some slight degradation of the protein once it is lyophilized and resuspended, as indicated by the bands seen at molecular weights less than 53 kDa, the molecular weight of F1-V; 2) None of the preparation/encapsulation techniques resulted in further degradation of F1-V; 3) There is no difference in the different nanoemulsion encapsulation methods as seen in comparing lanes 9 and 10; and 4) There is a significant reduction in the protein concentration between the initial liposome preparation (lane 7) and the supernatant after centrifugation (lane 8), indicating that a significant amount of F1-V is encapsulated in the vesicles (lane 6).

The formulations were then analyzed by Western blot; serum from mice immunized with F1-V was used for staining. A representative membrane is shown below:



The following components were loaded into each lane:
1) 2 µL ceramide 3 liposome supernatant
2) 1.5 µg F1-V in ceramide 3 liposome (initial preparation before ultracentrifugation) in a total volume of 2 µL
3) 15 µ L of liposome subnatant collected after ultracentrifugation
4) blank
5) 1.5 µg F1-V encapsulated in water-in-oil-in-water double emulsion formulation
6) blank
7) 0.75 µg F1-V in aqueous buffer
8) 1.5 µg F1-V in aqueous buffer
9) molecular weight marking standard

From these assays we confirmed that encapsulated F1-V protein retains its antigenicity (i.e., is recognized by mouse anti-F1-V sera). This indicates that the antigenicity of the protein is not hindered and that these formulations should be suitable for use in immunization studies to determine their ability to efficiently deliver antigen through intact skin for an appropriate anti-F1-V immune response.

KEY RESEARCH ACCOMPLISHMENTS

- Prepared twenty different formulations of poly(lactide-co-glycolide) (PLGA) micro/nanocapsules containing BSA.
- Optimized nanoemulsion formulation according to particle size, viscosity, and temporal stability.
- Developed five formulations of nanoemulsions containing FITC-BSA with an average particle size of less than 100 nm, which was ideal for permeation through skin.\

- Demonstrated that nanoemulsion formulations show consistent, superior results compared to larger particle size emulsion, demonstrating the impact of manufacturing technique and particle size on the efficacy of transcutaneous formulations.
- Demonstrated that encapsulation of a biologically relevant vaccine antigen (F1-V) in cermide-3 containing liposomes preserves the antigenicity of the protein and facilitates deliver across intact skin to the immune reactive cells in the epidermis.

REPORTABLE OUTCOMES

None

CONSLUSIONS

Encapsulating a vaccine antigen within or adsorbing it to appropriate nanocarriers should facilitate transport through the stratum corneum to the targeted dendritic cells of the epidermis and dermis to initiate an immune response. Tailoring the nanocarriers to optimize encapsulation and/or adsorption and permeation efficiency requires an understanding of the interactions between the molecules composing the carrier, the antigen of interest, and the skin components in addition to the potential immune response to the antigen and the possible effect of the carrier or coadministered adjuvants on this response. Antigen-presenting cells show more efficient uptake of antigen incorporated into or onto a vesicular or particulate carrier, suggesting the potential for nanocarriers to enhance not only transport of the antigen through the skin's barrier but also uptake of the antigen once it reaches the dendritic cells of the viable epidermis and dermis. Nanocarrier-based transcutaneous vaccines represent a promising application of nanotechnology for delivery of vaccines against biological threat agents. Moreover, the technologies created will have a tremendous impact on health and human welfare around the world because of their applicability to a wide range of infectious diseases and therapeutic treatments, including other infectious diseases that pose threats to the war-fighter.